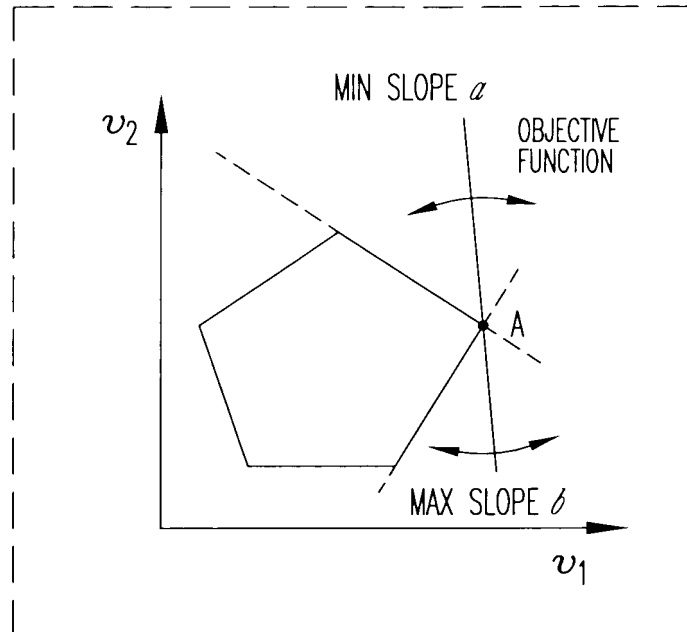
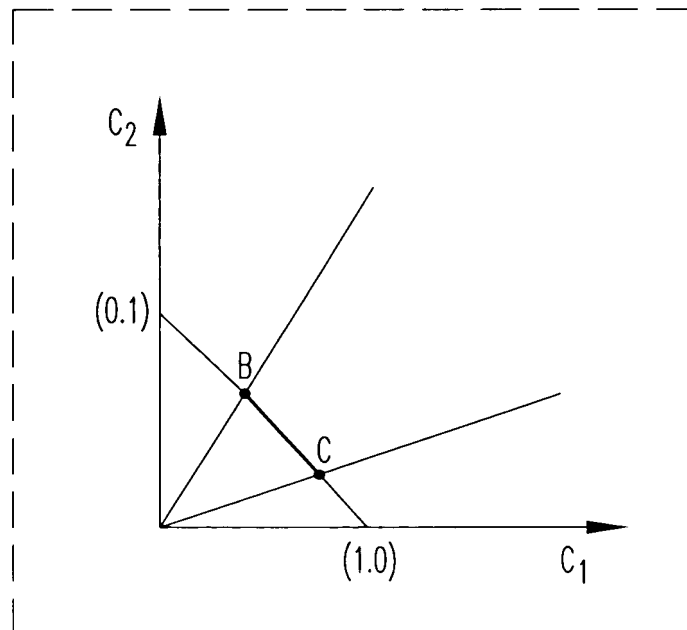


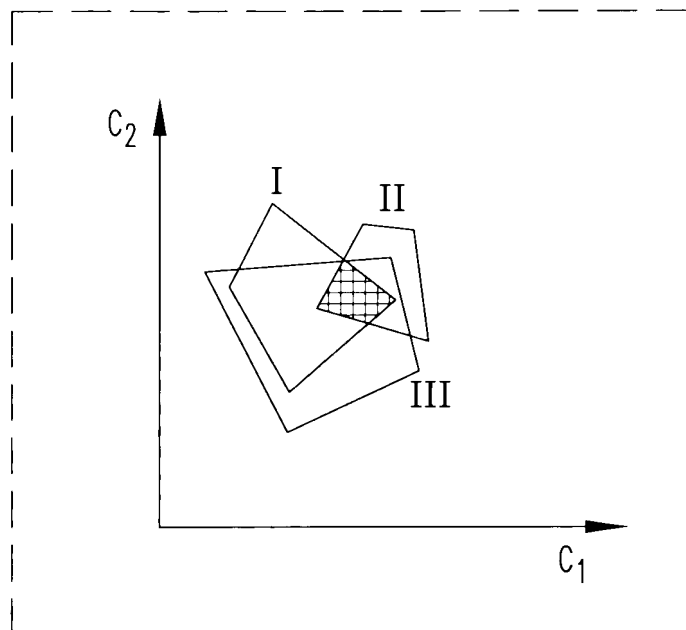
*Fig. 1*



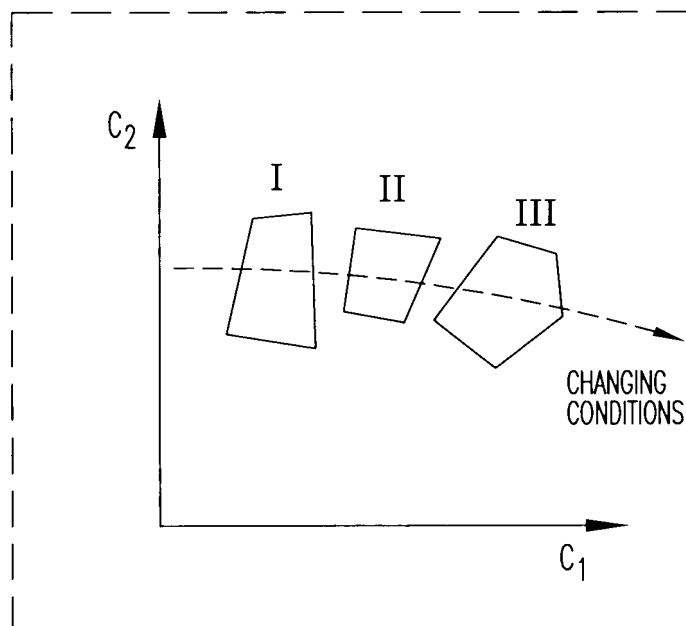
*Fig. 2A*



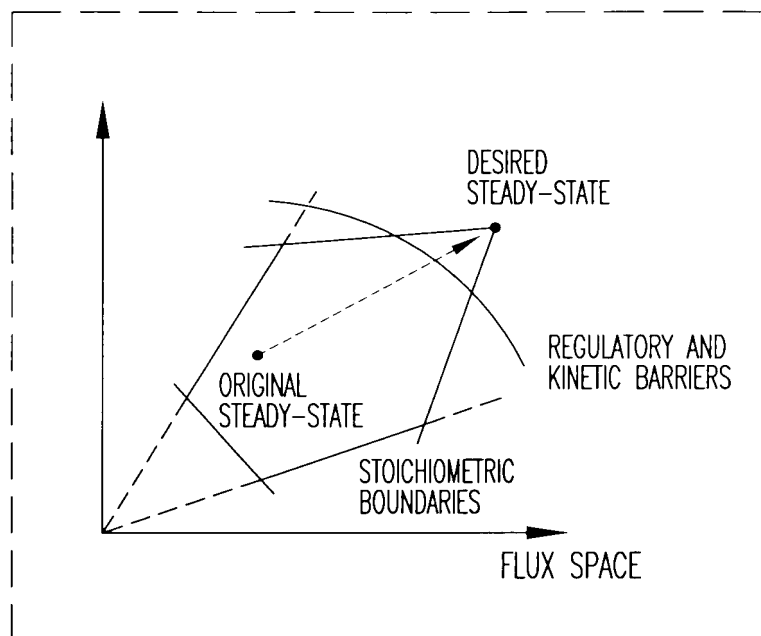
*Fig. 2B*



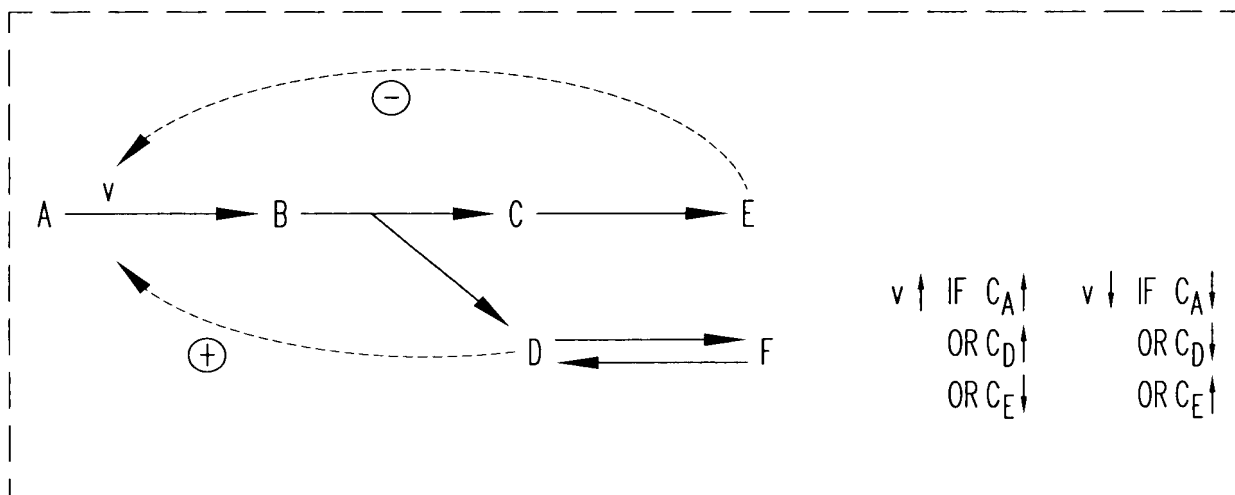
*Fig. 3A*



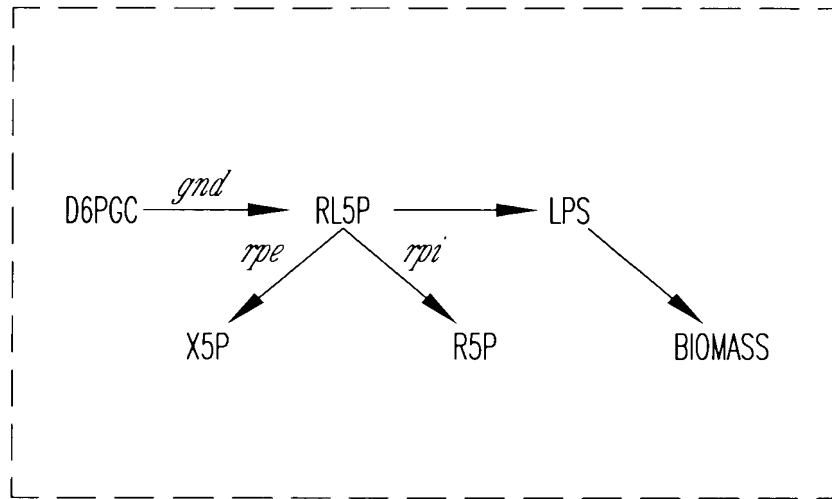
*Fig. 3B*



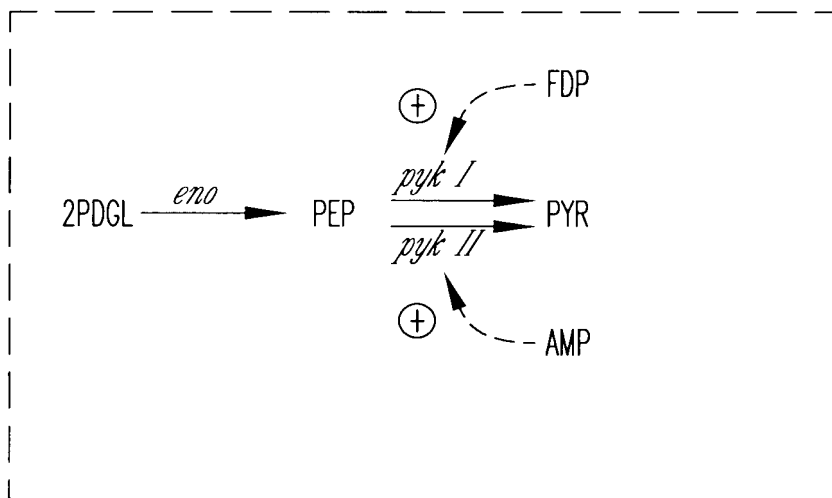
*Fig. 4*



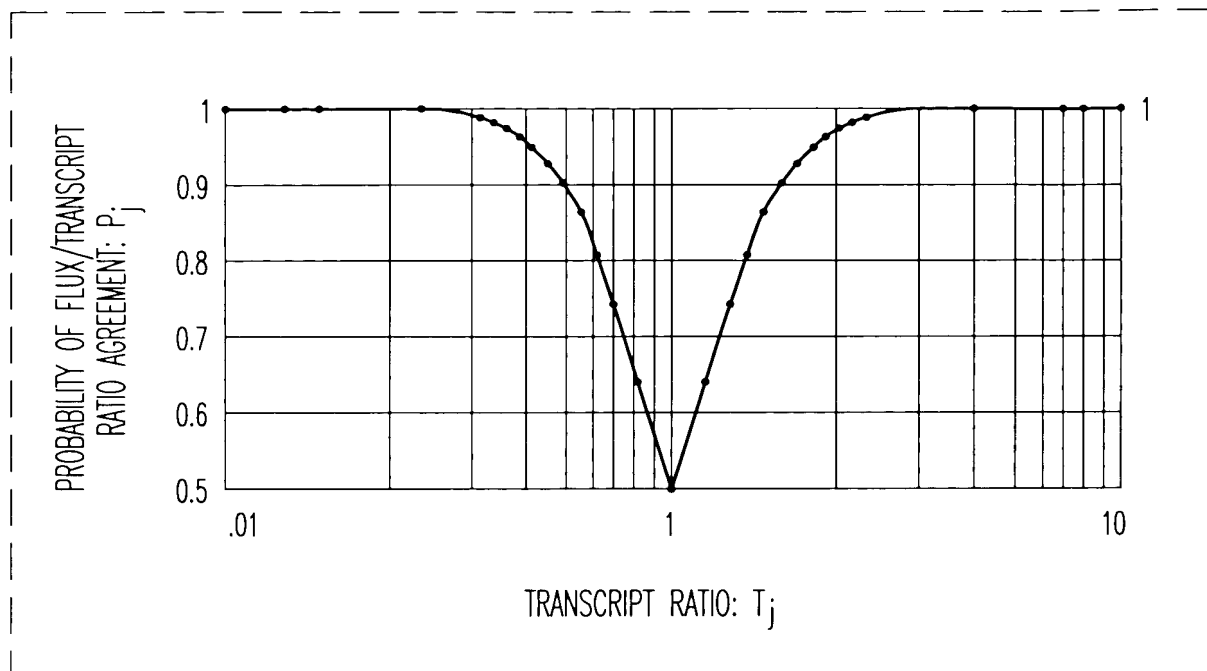
*Fig. 5*



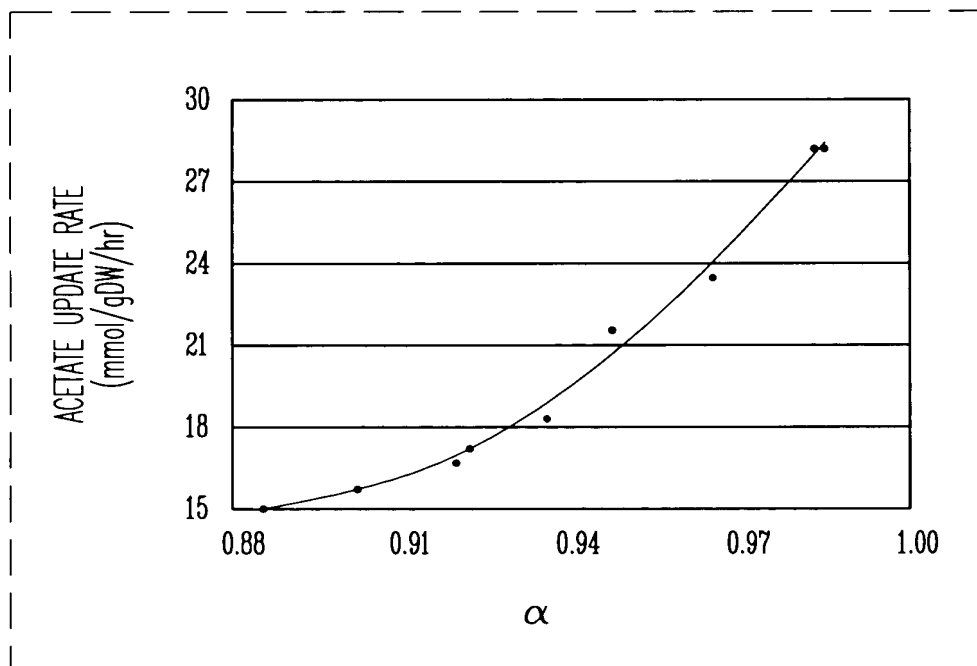
*Fig. 6A*



*Fig. 6B*



*Fig. 7*



*Fig. 8*

**MODEL PREDICTIONS**  
**OF MAXIMUM THEORETICAL YIELDS OF AMINO ACIDS FOR**  
**GROWTH ON GLUCOSE AND ACETATE**

	Maximum Theoretical Yield (mmol / per 10 mmol Glucose)				Maximum Theoretical Yield (mmol / per 10 mmol Acetate)			
	Palsson '93	Modified Keasling '97	Universal Model	% Increase	Palsson '93	Modified Keasling '97	Universal Model	% Increase
<b>Alanine</b>	20.00	20.00	20.00	-	3.93	5.29	5.29	-
<b>Arginine</b>	7.74	9.26	10.07	8.75%	1.51	2.43	2.65	9.05%
<b>Asparagine</b>	15.60	18.18	19.23	5.77%	3.24	4.66	4.91	5.45%
<b>Aspartate</b>	18.20	20.00	20.00	-	3.82	5.29	5.29	-
<b>Cysteine</b>	9.75	11.49	11.90	3.57%	1.81	3.29	3.42	3.80%
<b>Glutamate</b>	10.00	13.33	13.33	-	2.68	3.65	3.65	-
<b>Glutamine</b>	10.00	13.33	13.33	-	2.50	3.46	3.46	-
<b>Glycine</b>	20.00	35.33	35.33	-	3.94	9.00	9.00	-
<b>Histidine</b>	7.30	9.77	9.80	0.23%	1.37	2.43	2.54	4.53%
<b>Isoleucine</b>	7.34	8.00	8.07	0.91%	1.44	2.13	2.13	-
<b>Leucine</b>	6.67	8.00	8.00	-	1.59	2.18	2.18	-
<b>Lysine</b>	7.84	8.45	8.45	-	1.55	2.18	2.18	-
<b>Methionine</b>	5.74	7.04	7.19	2.16%	1.11	1.81	1.85	2.46%
<b>Phenylalanine</b>	5.29	5.76	5.76	-	1.00	1.47	1.47	-
<b>Proline</b>	10.00	10.91	10.91	-	2.10	2.90	2.90	-
<b>Serine</b>	20.00	23.04	23.04	-	3.94	5.87	5.87	-
<b>Threonine</b>	12.30	15.00	15.00	-	2.50	3.91	3.91	-
<b>Tryptophan</b>	4.14	4.67	4.73	1.28%	0.76	1.17	1.19	1.32%
<b>Tyrosine</b>	5.48	6.03	6.03	-	1.03	1.54	1.54	-
<b>Valine</b>	10.00	10.00	10.00	-	1.96	2.67	2.67	-

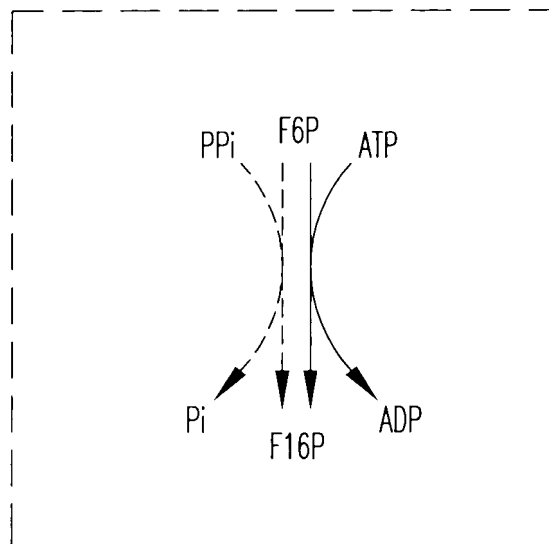
Palsson '93: *E. coli* model proposed by Palsson (1993)

Modified Keasling '97: Modified Keasling (1997) *E. coli* model as described in text

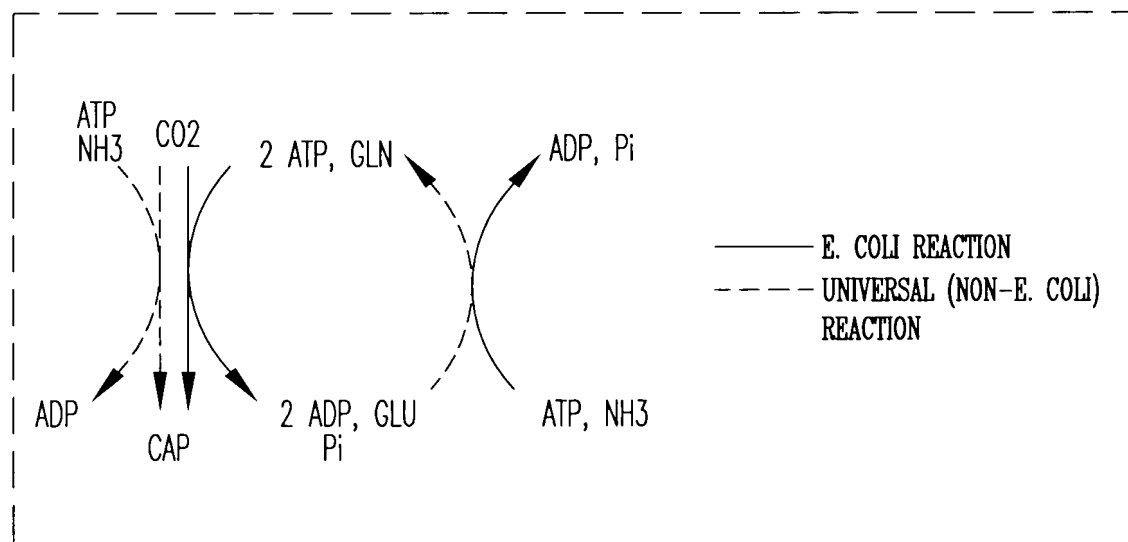
Universal Model: Modified Keasling (1997) *E. coli* model augmented with non-*E. coli* reactions compiled by the Kyoto Encyclopedia of Genes and Genomes

% Increase: Between the modified Keasling (1997) model and the Universal model

**Fig. 9**

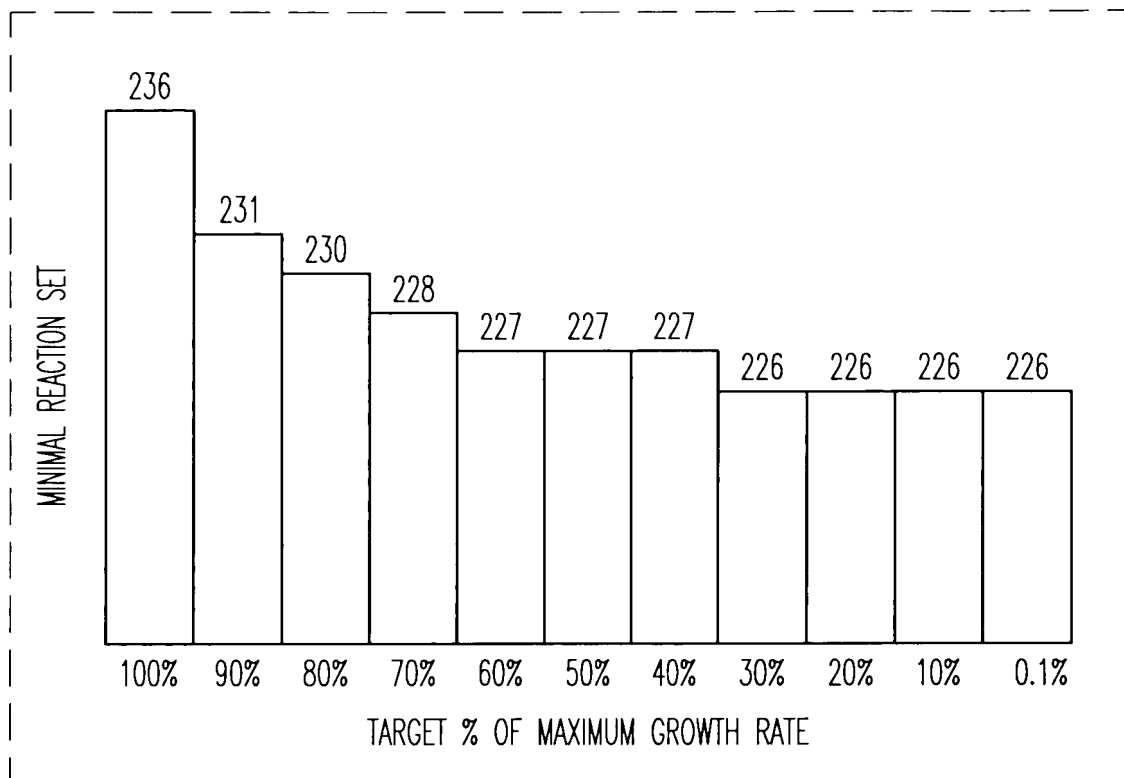


*Fig. 10A*

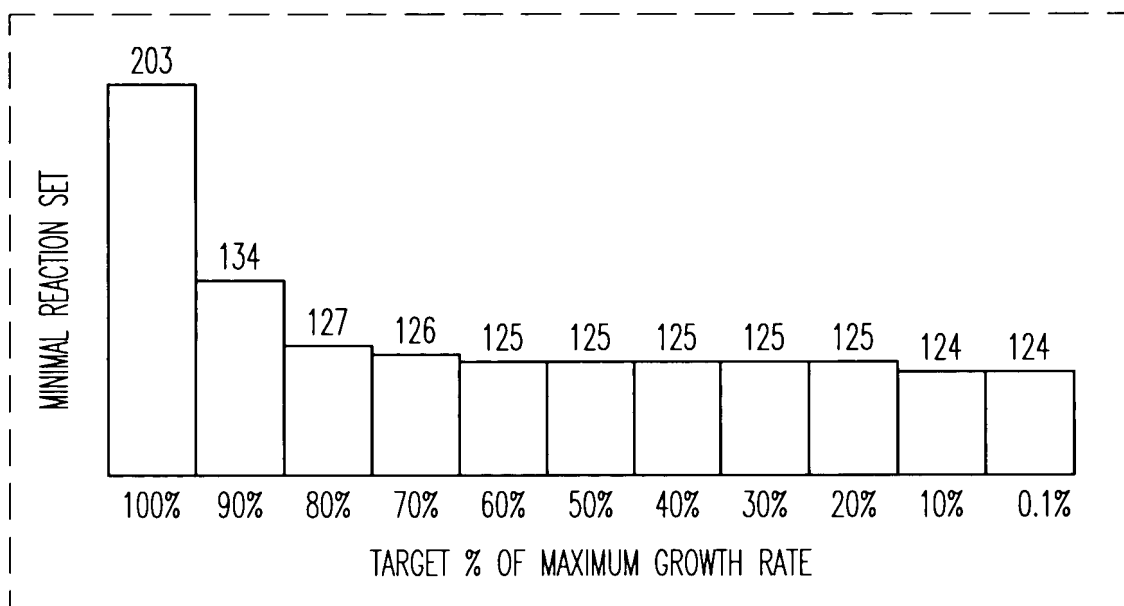


*Fig. 10B*





*Fig. 11A*



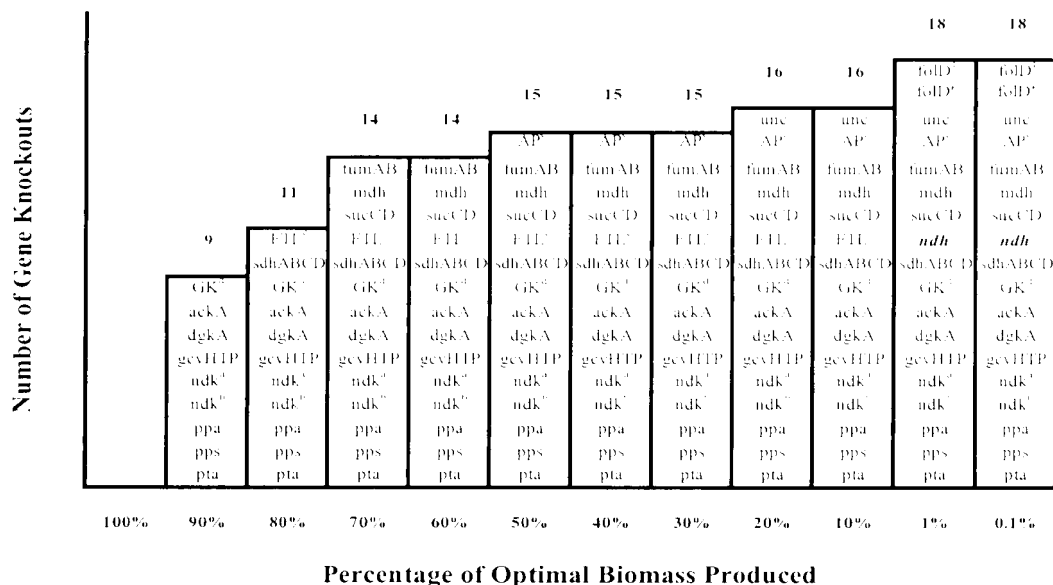
*Fig. 11B*

## MODIFICATIONS TO THE PRAMANIK AND KEASLING MODEL \*

Enzymes	Reactions
<b>Reactions assumed irreversible</b>	
Phosphofructokinase	Fructose-1,6-bisphosphate $\rightarrow$ Fructose-6-phosphate + Pi
Citrate Synthase	Acetyl-CoA + Oxaloacetate $\rightarrow$ CoA + Citrate
2-Ketoglutarate Dehydrogenase	2-Ketoglutarate + NAD + CoA $\rightarrow$ Succinyl-CoA + CO <sub>2</sub> + NADH
PRSCAIM Synthetase	RCAIM + ATP + Aspartate $\rightarrow$ ADP + Pi + PRSCAIM
Glycerol Kinase	Glycerol + ATP $\rightarrow$ Glycerol-3-phosphate + ADP
<b>Reactions removed from model</b>	
Unknown Pathway	5'-methylthioadenosine $\rightarrow$ Adenosine + Methionine
Cystathionase	Homocysteine + Adenosine $\leftrightarrow$ s-Adenosyl-homocystine
Sulfotransferase	Adenosine-3,5-diphosphate + sulfite $\leftrightarrow$ 3-Phosphoadenylylsulfate
<b>Reactions modified</b>	
Fructose-1,6-bisphosphate Aldolase	Fructose-1,6-bisphosphate $\rightarrow$ Fructose-6-phosphate + Pi
Isocitrate Dehydrogenase	Isocitrate + NADP $\leftrightarrow$ CO <sub>2</sub> + NADPH + 2-Ketoglutarate
Succinate Thiokinase	Succinyl-CoA + ADP + Pi $\leftrightarrow$ ATP + CoA + Succinate
Prephenate Dehydrogenase	Prephenate + NAD $\rightarrow$ CO <sub>2</sub> + NADH + para-Hydroxy phenyl pyruvate
Hol Dehydrogenase	Histidinol + 3 NAD $\rightarrow$ 3 NADH + Histidine
RCAIM Synthetase	AIR + CO <sub>2</sub> + ATP $\rightarrow$ 5-p-Ribosyl-4-carboxy-5-aminoimidazole + ADP + Pi
GTP Cyclohydrolase	GTP $\rightarrow$ D6RP5P + Formate + Ppi
3,4-Dihydroxy-2-Butanone-4-Phosphate Synthase	Ribulose-5-phosphate $\rightarrow$ DB4P + Formate
H2Neopterin Triphosphate	AHTD $\rightarrow$ Ppi + Pi + DHP
Pyrophosphatase	
CoA Synthase	OIVAL + METTHF + NADPH + ALA + CTP + 4 ATP + CYS $\rightarrow$ THF + NADP + AMP + 2 Ppi + 2 ADP + CO <sub>2</sub> + CoA + CDP

## MODIFICATIONS BASED ON INFORMATION BY KARP (1999)

*Fig. 12*



<sup>ab</sup> Same gene responsible for two intracellular reactions  
<sup>bc</sup> Same gene responsible for two intracellular reactions  
<sup>cde</sup> No gene has been assigned to these intracellular reactions

**Fig. 13**

## GENES SELECTED FOR REMOVAL BY KNOCKOUT STUDY

Enzymes	Genes	Reactions
3,5-ADP Phosphatase	AP <sup>e</sup>	35ADP → AMP + Pi
Acetate Kinase	ackA	AC + ATP → ACTP + ADP
CDP Kinase	ndk <sup>a</sup>	CDP + ATP → CTP + ADP
CMP Kinase	ndk <sup>b</sup>	CMP + ATP → CDP + ADP
F0F1-ATPase	unc	ADP + Pi + H <sub>ext</sub> → ATP
Formate THF Ligase	FTL <sup>c</sup>	THF + FORMATE + ATP → ADP + Pi + FTHF
Fumarase	fumAB	FUM → MAL
Glyceraldehyde Kinase	GK <sup>d</sup>	GLAL + ATP → ADP + T3P1
Glycine Cleavage System	gcvHTP	GLY + THF + NAD → METTHF + NADH + CO2 + NH3
Malate Dehydrogenase	mdh	MAL + NAD → NADH + OA
Methenyl THF Cyclohydrolase	folD <sup>f</sup>	METHF → FTHF
Methylene THF Dehydrogenase	folD <sup>g</sup>	METTHF + NADP → METHF + NADPH
NADH Dehydrogenase I	ndh	NADH + Q → NAD + QH2 + 4 H <sub>ext</sub>
PEP Synthase	pps	PYR + ATP → PEP + AMP + Pi
Phosphatidate Phosphatase	dgkA	DGR + Pi → PA
Phosphotransacetylase	pta	ACTP + COA → ACCOA + Pi
Pyrophosphatase	ppa	PPi → 2 Pi
Succinate Dehydrogenase	sdhABCD	SUCC + FAD → FADH2 + FUM
Succinate Thiokinase	sucCD	SUCCOA + GDP + Pi → GTP + COA + SUCC

a,b Same gene responsible for two intracellular reactions

f,g Same gene responsible for two intracellular reactions

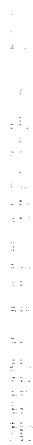
c,d,e No gene has been assigned to these intracellular reactions

*Fig. 14*

**MODEL SELECTIONS OF ENZYMATIC REACTIONS THAT WILL  
ENHANCE THE AMINO ACID PRODUCTION CAPABILITIES OF  
*ESCHERICHIA COLI***

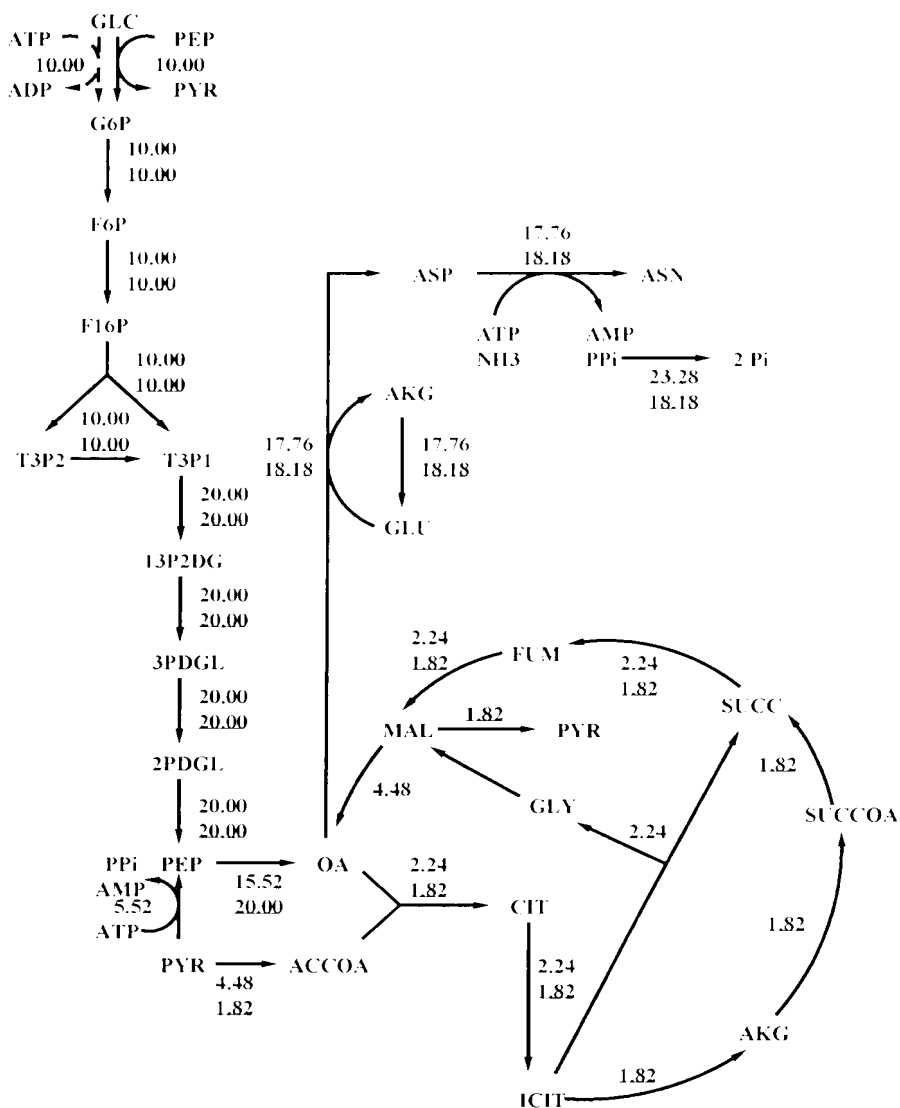
Amino Acid	Substrate	EC#	Enzyme	Reaction Catalyzed
<b>Arginine</b>	Glucose:	2.7.1.90	6-Phosphofructokinase (pyrophosphate)	Fructose-6-P + PPi → Fructose-1,6-Bisphosphate + Pi
		2.7.2.2	Carbamate kinase	ATP + NH <sub>3</sub> + CO <sub>2</sub> → ADP + Carbamoyl Phosphate
	Acetate:	2.7.2.2	Carbamate kinase	ATP + NH <sub>3</sub> + CO <sub>2</sub> → ADP + Carbamoyl Phosphate
		2.7.2.12	Acetate kinase (pyrophosphate)	Acetate + PPi → Pi + Acetyl-Phosphate
<b>Asparagine</b>	Glucose/ Acetate:	5.3.1.4	Aspartate—ammonia ligase (ADP- forming)	ATP + NH <sub>3</sub> + L-Aspartate → Pi + ADP + L-Asparagine
<b>Cysteine</b>	Glucose/ Acetate:	2.7.7.5	Sulfate adenyltransferase (ADP)	Sulfate + ADP → Pi + Adenyl-Sulfate
<b>Histidine</b>	Glucose:	1.4.1.10	Glycine dehydrogenase	NAD + glycine → glyoxylate + NADH + NH <sub>3</sub>
		2.7.1.90	6-Phosphofructokinase (pyrophosphate)	Fructose-6-P + PPi → Fructose-1,6-Bisphosphate + Pi
	Acetate:	1.4.1.10	Glycine dehydrogenase	NAD + glycine → glyoxylate + NADH + NH <sub>3</sub>
		4.1.1.38	Phosphoenolpyruvate carboxykinase (pyrophosphate)	PPi + Oxaloacetate → CO <sub>2</sub> + Pi + PEP
<b>Isoleucine</b>	Glucose:	many		
<b>Methionine</b>	Glucose:	2.7.7.5	Sulfate adenyltransferase (ADP)	Sulfate + ADP → Pi + Adenyl-Sulfate
	Acetate:	1.4.1.10	Glycine dehydrogenase	NAD + glycine → glyoxylate + NADH + NH <sub>3</sub>
		2.7.7.5	Sulfate adenyltransferase (ADP)	Sulfate + ADP → Pi + Adenyl-Sulfate
		2.7.9.1	Pyruvate,phosphate dikinase	Pyruvate + Pi + ATP → AMP + PPi + PEP
		4.1.1.38	Phosphoenolpyruvate carboxykinase (pyrophosphate)	PPi + Oxaloacetate → CO <sub>2</sub> + Pi + PEP
<b>Tryptophan</b>	Glucose:	2.7.1.90	6-Phosphofructokinase (pyrophosphate)	Fructose-6-P + PPi → Fructose-1,6-Bisphosphate + Pi
		2.7.9.1	Pyruvate,phosphate dikinase	Pyruvate + Pi + ATP → AMP + PPi + PEP
	Acetate:	2.7.9.1	Pyruvate,phosphate dikinase	Pyruvate + Pi + ATP → AMP + PPi + PEP
		4.1.1.38	Phosphoenolpyruvate carboxykinase (pyrophosphate)	PPi + Oxaloacetate → CO <sub>2</sub> + Pi + PEP

**Fig. 15**



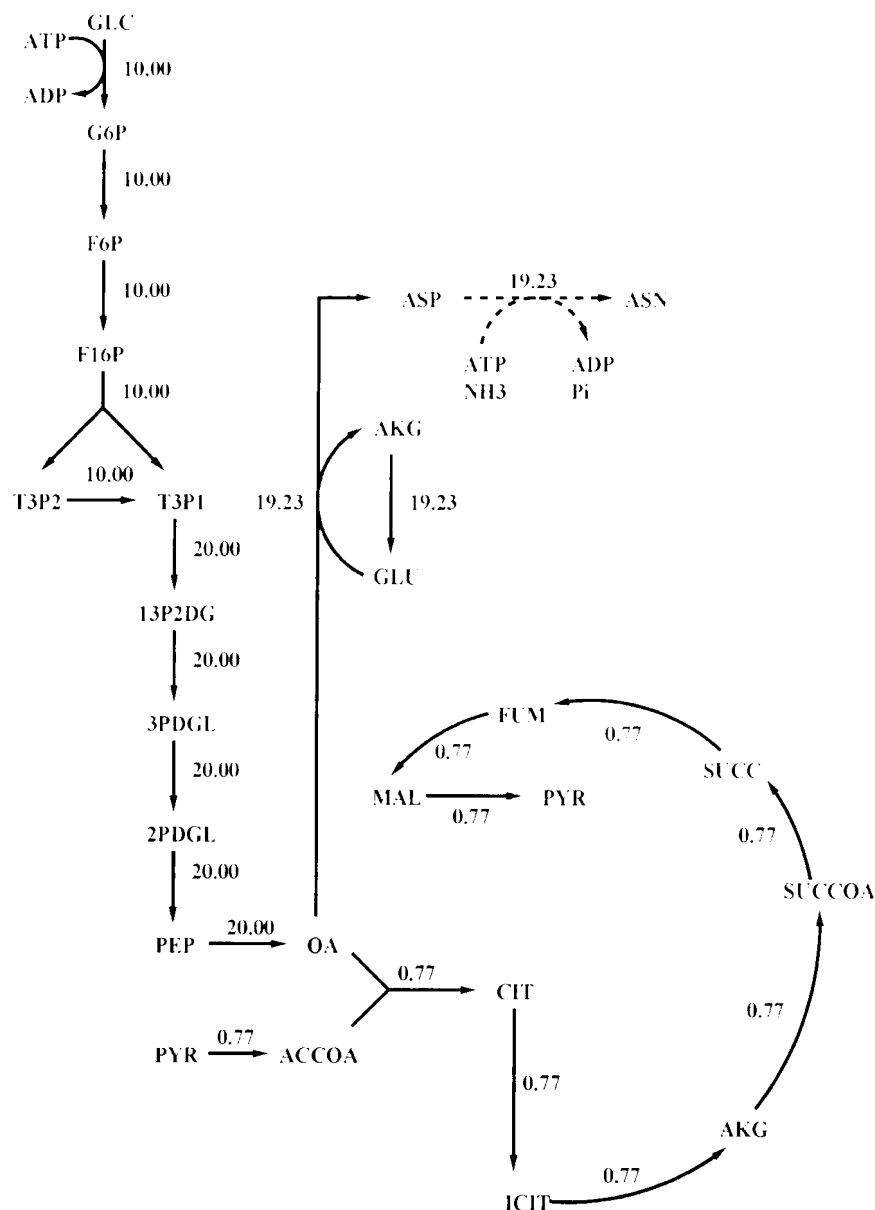
*Fig. 16*



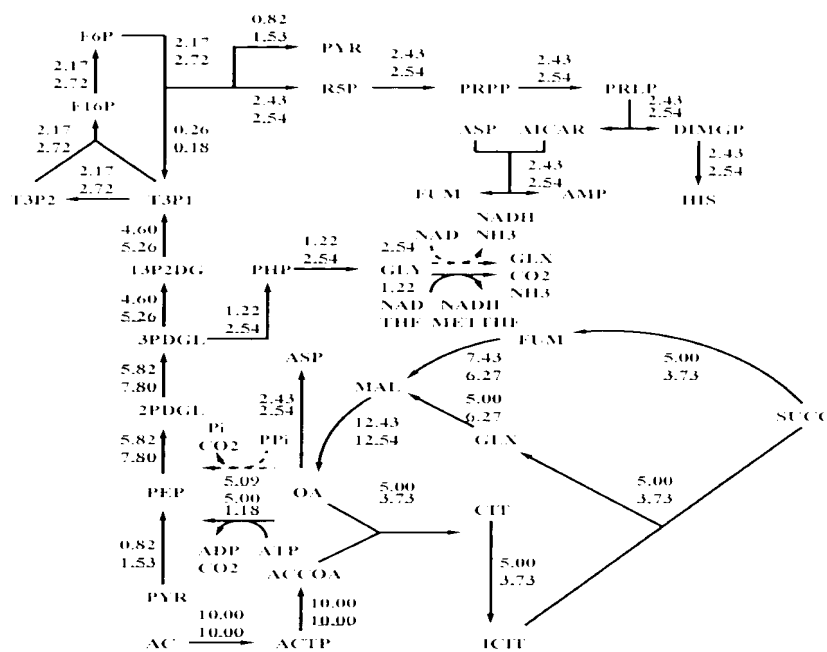


*Fig. 18*

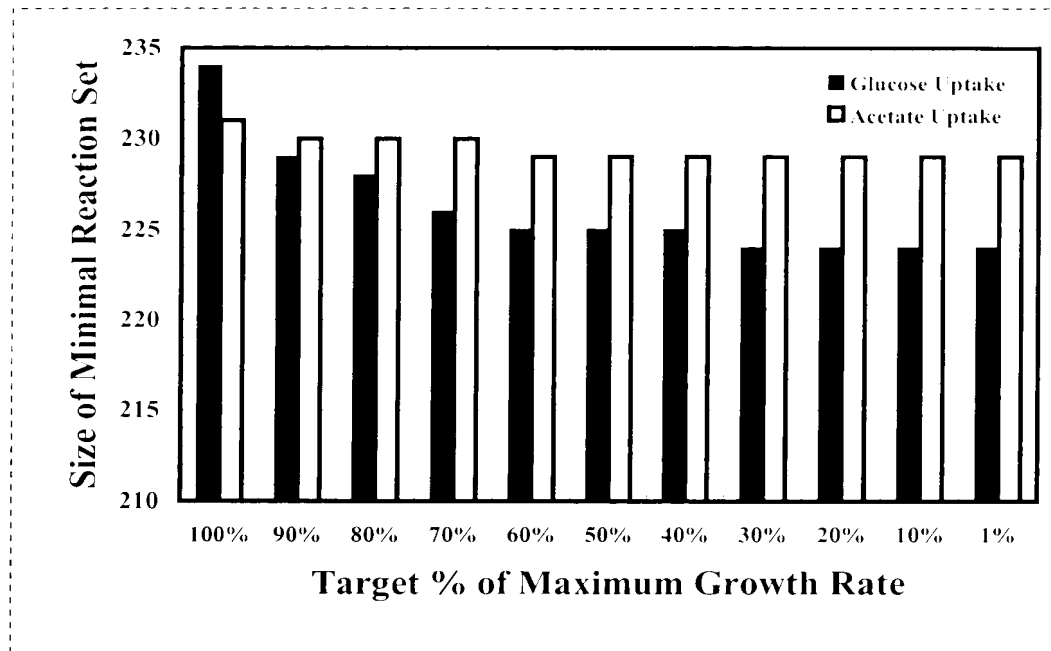




*Fig. 19*



*Fig. 20*



*Fig. 21*

**EVOLUTION OF MINIMAL REACTION SETS FOR CASE (I)  
UNDER DECREASING GROWTH REQUIREMENTS.**

<b>Target % Maximum Growth Rate</b>	<b>Minimal Reaction Set (# Reactions)</b>	<b>Key Features</b>
100%	234	The glycolysis, tricarboxylic acid cycle, and pentose phosphate pathways are all operating in their forward directions, optimally generating the energy cofactors ATP, NADH, and NADPH required for cell growth. All available glucose is oxidized into the cell's only secreted byproduct, carbon dioxide.
90%	229	The fluxes through two TCA cycle reactions 2-ketoglutarate dehydrogenase and succinate dehydrogenase are zero while succinyl-CoA synthetase operates in its reverse direction suggesting a less demanding energetic state under the sub-maximal growth demands. Acetate is now secreted as a byproduct along with carbon dioxide.
80%	228	Fluxes through two additional TCA cycle reactions, fumarase and malate dehydrogenase, are eliminated while a reaction secreting fumarate is added.
70%	226	The pentose phosphate pathway operates solely for nucleotide biosynthesis with the reaction fluxes through ribulose phosphate 3-epimerase, transketolase I, transketolase II, and transaldolase B all operating in reverse. Fluxes through glucose-6-phosphate dehydrogenase, lactonase, and 6-phosphogluconate dehydrogenase are absent in this case, replaced by pyridine nucleotide transhydrogenase which meets the cellular NADPH needs. In addition, formate is now secreted along with acetate, fumarate, and carbon dioxide.
60%, 50%, 40%	225	Acetate is no longer secreted as a metabolic byproduct, but is converted to acetyl-CoA by acetyl-CoA synthetase.
30%, 20%, 10%, 1%	224	Three glycolytic reactions, phosphoglycerate mutase, enolase, and pyruvate kinase are eliminated, but both serine deaminase and phosphoenolpyruvate synthase are added to supply the cell with phosphoenolpyruvate.

**Fig. 22**

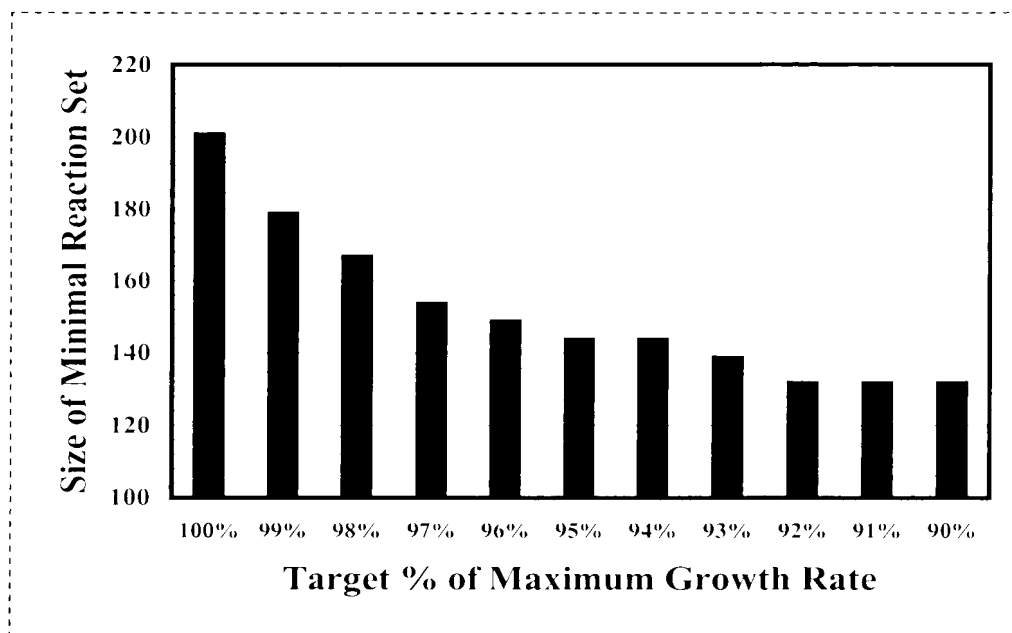
**METABOLITES UPTAKEN OR SECRETED AT EACH TARGET GROWTH  
RATE ON AN OPTIMALLY ENGINEERED MEDIUM.**

**U DENOTES METABOLITE UPTAKE**

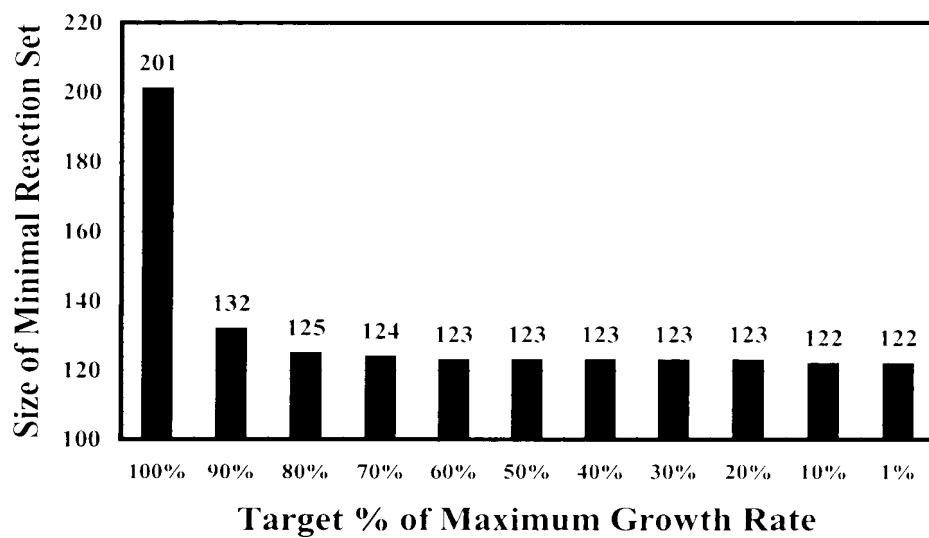
**S DENOTES METABOLITE SECRETION**

Metabolite	Percentage of 100% Biomass Generation Required													
	100%	99.5%	99%	98%	97%	96%	95%	90%	85%	80%	70%	60%	10%	
Acetate												S	S	
Acetaldehyde													U	
Adenine				U	U	U	U	U	U			U		
Adenosine										U	U		U	
Alanine										U	U			
Arginine	U	U	U	U	U	U	U	U	U	U	U	U	U	
Asparagine									U	U	U	U	U	
Aspartate									U	U	U	U	U	
Carbon dioxide	S	S	S	S	S	S	S	S	S	S	S	S	S	
Cysteine	U	U	U	U	U	U	U	U	U	U	U	U	U	
D-Alanine								U	U			U	U	
Thymidine		U	U	U	U	U	U	U	U	U	U	U	U	
Ethanol	U	U	U	U	U	U	U	U		U		U		
Glycerol											U			
Glycerol-3-phosphate	U	U	U	U	U	U	U	U	U	U		U	U	
Glutamine									U	U	U	U	U	
Glutamate											S	U	U	
Glycine						U	U	U	U	U	U	U	U	
Guanine				U	U	U	U		U	U				
Guanosine								U			U	U	U	
Histidine		U	U	U	U	U	U	U	U	U	U	U	U	
Isoleucine	U	U	U	U	U	U	U	U	U	U	U	U	U	
Leucine							U	U	U	U	U	U	U	
Lysine	U	U	U	U	U	U	U	U	U	U	U	U	U	
Meso-diaminopimelate		U	U	U	U	U	U	U	U	U	U	U	U	
Methionine	U	U	U	U	U	U	U	U	U	U	U	U	U	
Mannitol												U	U	
Ammonia	U	U	U	U	U	U	U	U						
Oxygen	U	U	U	U	U	U	U	U	U	U	U	U	U	
Phenylalanine			U	U	U	U	U	U	U	U	U	U	U	
Phosphate	U	U	U	U	U	U	U	U	U	U	U	U	U	
Proline					U	U	U	U	U	U	U	U	U	
Putrescine	U	U	U	U	U	U	U	U	U	U	U	U	U	
Pyruvate										U	U	U	U	
Ribose												U	U	
Serine								U	U	U	U	U	U	
Spermidine	U	U	U	U	U	U	U	U	U	U	U	U	U	
Threonine		U	U	U	U	U	U	U	U	U	U	U	U	
Tryptophan		U	U	U	U	U	U	U	U	U	U	U	U	
Tyrosine			U	U	U	U	U	U	U	U	U	U	U	
Uracil						U	U	U	U	U		U		
Uridine											U	U	U	
Valine							U	U	U	U	U		U	
# Metabolites Uptaken	12	17	19	21	22	24	26	28	29	31	29	34	34	

**Fig. 23**



*Fig. 24*



*Fig. 25*

**EVOLUTION OF MINIMAL REACTION SETS FOR CASE (II)  
UNDER DECREASING GROWTH REQUIREMENTS.**

<b>Target % Maximum Growth Rate</b>	<b>Minimal Reaction Set (# Reactions)</b>	<b>Key Features</b>
100%	201	The organic material transported into the cell includes ethanol and glycerol-3-phosphate which fuel glycolysis, the TCA cycle, and PPP. The flux directions of the glycolysis pathway are split with all reaction fluxes preceding glyceraldehyde-3-phosphate (G3P) dehydrogenase operating in reverse, and all fluxes following and including G3P dehydrogenase operate in their forward directions. Putrescine, spermidine, and five amino acids are transported into the network eliminating the need for biosynthetic pathways for these components.
90%	132	While the PPP and TCA cycle reactions are still functional, the network no longer utilizes the five glycolytic reactions from glyceraldehyde-3-phosphate dehydrogenase to pyruvate kinase. Consequently, the TCA cycle is completely fueled by imported ethanol and acetate rather than flux from the glycolysis pathway.
80%	125	This network tolerates the complete elimination of the TCA cycle and glyoxylate shunt. As a result, the function of the pentose phosphate pathway reactions is no longer restricted to nucleotide biosynthesis, but now includes the formation of cellular NADPH. Most of this NADPH is subsequently converted to NADH by pyridine nucleotide transhydrogenase to replace the cellular reducing power lost from the inactivity of the TCA cycle.
70%	124	A slightly less efficient set of internal metabolic reactions enables the growth demands to be met with the importation of one less metabolite (i.e. one less transport reaction) than its 80% counterpart.
60% 50%, 40% 30%, 20%	123	Neither the TCA cycle nor PPP are utilized for reducing power. Most of the cellular reducing capabilities are now generated from the uptake of ethanol and its subsequent conversion into acetyl-CoA.
10%, 1%	122	This minimal network is comprised mostly of cell envelope and membrane lipid biosynthetic reactions, along with a number of transport and salvage pathway reactions. Here, the three core metabolic routes, glycolysis, the TCA cycle, and the pentose phosphate pathway are almost completely dismantled with only one glycolytic and 4 PPP reactions remaining.

**Fig. 26**



FUNCTIONAL CLASSIFICATION OF MINIMAL  
NETWORK REACTIONS FOR GROWTH ON AN OPTIMALLY  
ENGINEERED MEDIUM.

Functional Classification	# rxns
ALA Isomerization	1
Alternative Carbon Source	7
Anaplerotic Reactions	1
Cell Envelope	
Biosynthesis	29
EMP Pathway	5
Membrane Lipid	
Biosynthesis	16
Pentose Phosphate	
Pathway	4
Pyrimidine Biosynthesis	1
Respiration	5
Salvage Pathways	17
Transport	36
	<b>122</b>

*Fig. 27*

**COMPARISON OF MINIMAL METABOLIC GENE/REACTION  
SETS BASED ON FUNCTIONAL CLASSIFICATION\***

<b>Metabolic Function</b>	<b>Essential Gene Set Ref. (2)</b>	<b>Minimal Gene Set Ref. (5)</b>	<b>Minimal Reaction Set</b>
	<b># Genes</b>	<b># Genes</b>	<b># Reactions</b>
Amino acid biosynthesis	0	0	1
Biosynthesis of cofactors, prosthetic groups, and carriers	4	3	0
Cell envelope	2	11	29
Central intermediary metabolism	7	7	1
Energy metabolism	31	32	21
Fatty acid and phospholipid metabolism	5	7	16
Purines, pyrimidines, nucleosides, and nucleotides	17	14	18
Transport and binding proteins	17	25	36
	<b>83</b>	<b>99</b>	<b>122</b>

*Fig. 28*